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OF BOTULINUM NEUROTOXINS

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19. ABSTRACT (Continue on reverse if necessary and identify by block number) With the aim of identifying domains in botulinum neurotoxin (BoNT) responsible for blockade of transmitter release, a prerequisite for developing treatments for botulism, the heavy (HC) and light (LC) chains of types A and B were purified to homogeneity, renatured, or reconstituted to give a di-chain toxic species; controlled proteolysis of BoNT A followed by chromatographic procedures yielded pure fragments [H₂L, intact toxin minus the C-terminal half of HC (H₁); H₂, N-terminal portion of HC]. These were tested, alone or in combination, for inhibitory effects on neurally-evoked transmitter release from mouse nerve diaphragm and Aplysia ganglionic neurons. The latter allowed extra- or intra-neuronal administration of toxin samples, with quantitation of quantal release by voltage-clamp analysis of pairs of pre- and post-synaptic cells. Examination of an intracellular action of BoNT was, also, accomplished by digitonin-permeabilisation of cultured PC-12 cells, with retention of the exocytosis process. For monitoring the toxin's molecular action, nerve terminals or synaptic vesicles were isolated from brain.

By means of these multi-disciplinary approaches, the following results were obtained:

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18. SUBJECT TERMS
line; PC-12 cells; cell permeabilisation; G-proteins; inhibition of neurotransmitter release.

19. ABSTRACT

(1) Following external application to motor nerves or *Aplysia* ganglia, BoNT blocked efficiently transmitter release from cholinergic nerve terminals *only*, by a multi-phasic mechanism involving binding to ecto-acceptors (absent from other cells), acceptor-mediated uptake and an undefined intra-neuronal step. (2) Upon by-passing the uptake stage, BoNT A or B inactivated a ubiquitous, intracellular component of the Ca^{2+}-dependent release process in both cholinergic and non-cholinergic neurons of *Aplysia*, and in PC-12 cells. (3) This action required both chains inside *Aplysia* neurons, whereas LC alone seemed adequate in PC-12; acetylcholine release from the invertebrate cells was inhibited by intra-neuronal injection of H_2L or LC but *only* when HC was present, high-lighting an intra-cellular role for H_1 particularly because a mixture of LC and H_2 proved ineffective. (4) Investigation of the uptake step showed that H_2L or HC (but not LC) were internalised in cholinergic neurons of *Aplysia* and either HC or H_2 could mediate the translocation of LC; at the neuromuscular junction addition of HC with LC resulted in neuroparalysis but with low potency, consistent with the virtual inability of HC to antagonise the action of intact BoNT. These findings demonstrate that the targetting/uptake process at motor nerve endings is mediated by H_1 region *plus* structural features (including H_2) present in the di-chain toxin species whereas H_2 alone can serve this role in *Aplysia*, though probably less efficiently. (5) No evidence was obtainable for involvement of phosphorylation/dephosphorylation or ADP-ribosylation of in the action of BoNT A or B; G-proteins with M_r ∿25-27 kD and, apparently, ADP-ribosylated with partially-pure type D toxin were identified in synaptosomes and synaptic vesicles but the significance of these to the toxin's inhibition of exocytosis remains unsubstantiated.

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

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Abbreviations: BoNT A, B, D and E, botulinum neurotoxin types; HC and LC, heavy and light chains of BoNT; H_2L, intact BoNT A minus the C-terminal half of HC; H_2, N-terminal portion of HC; MPSC, miniature postsynaptic current; ACh, acetylcholine; KGEP, 139mM K^+ glutamate, 20mM Pipes pH 6.6, 1mM Mg^{2+}-ATP, 5mM EGTA, 5mM glucose and 0.5mM ascorbic acid.

INTRODUCTION

A major aim of this contract is to devise new treatments for botulism, a fatal neuroparalytic condition in man, caused by several types of botulinum neurotoxin (BoNT) - large proteins (M_r ~150,000, produced by <u>Clostridium botulinum</u> strains and consisting of a heavy chain (HC; M_r ~95,000) disulphide-linked to a light (LC; M_r ~55,000). The experimental strategy adopted involves:

(a) Establishing <u>conclusively</u> that BoNT blocks transmitter release from nerve terminals by a multi-phasic mechanism (ie. targetting/binding to ecto-acceptors unique to cholinergic nerves, acceptor-mediated uptake and inactivation of an intra-neuronal component concerned with the Ca^{2+}-dependent secretion process;

(b) Identifying the toxin's chains or domains responsible for each of these steps and

(c) Preparing antibodies against such pinpointed structures that would antagonise all phases of the intoxication.

Upon embarking on the project, it was known that BoNT type A when applied externally inhibits acetylcholine (ACh) release almost exclusively in the peripheral nervous system (reviewed by Dolly <u>et al.</u>, 1986). Accordingly, saturable binding of ^{125}I-labelled BoNT A or B to ecto-acceptors followed by internaliza-tion could be observed autoradiographically on cholinergic but not other nerve types (Black and Dolly, 1986a,b, 1987). However, the relevance of such an effective uptake system remained unsubstantiated because there was no direct evidence then for an intra-neuronal action of the toxin, though deductions drawn from pharmacological studies at the neuromuscular junction favoured this (reviewed by Simpson, 1986). Moreover, the latter seemed to underlie the susceptibility of peripheral cholinergic neurons to BoNT since it reduces Ca^{2+}-dependent efflux of ACh and several other transmitters from brain synaptosomes (reviewed by Dolly <u>et al.</u>, 1987) provided high concentrations are employed to overcome the lack of an efficient uptake; also, this could explain the low toxicity of BoNT when applied directly into brain (Williams <u>et al.</u>, 1983). Regarding identity of functional domains in the

toxin concerned with targetting/internalization, electron-microscopy studies at motor nerve terminals revealed that isolated HC could prevent the binding and subsequent uptake of ^{125}I-BoNT A (Black and Dolly, 1986b), albeit at relatively high concentrations. In contrast, there were reports of HC blocking BoNT binding to cerebrocortical synaptosomes with equal efficacy to the intact molecule (Kozaki, 1979; Williams et al., 1983). Notably, the C-terminal half of HC (H_1 fragment) contributes to this interaction with acceptor sites because the proteolytically-prepared H_2L fragment (intact toxin minus H_1) is unable to antagonise binding of ^{125}I-BoNT A to brain synaptosomes (Shone et al., 1985). Furthermore, the abilities of HC or its N-terminal moiety (H_2 fragment) to form pores in artificial membranes implicated part of this chain in the uptake step (Donovan and Middlebrook, 1986; Shone et al., 1987). In view of such encouraging findings with these various experimental systems, it is imperative to ascertain if all the toxin fragments exhibit the same pattern of activities on cholinergic nerve terminals and, particularly, whether the binding and uptake phenomena observed underlie the intoxication. Thus, a neuromuscular junction preparation, the prime target of BoNT, was used to quantify the abilities of the toxin's chains and available fragments (alone and in combination) to block nerve-evoked twitch tension or to antagonise the neuroparalytic effect of the intact toxin. Additionally, the samples were tested on large neurons in Aplysia ganglia because these offered major advantages for investigating the binding, uptake and intra-cellular action in that toxin could be applied externally and/or internally to cholinergic (or non-cholinergic) cells with electrophysiological recording of evoked quantal release of transmitters. To allow comparative investigations of the toxin's intra-cellular action in mammalian-derived cells, effects of the toxin preparations on Ca^{2+}-induced release of noradrenaline from cultured pheochromocytoma (PC-12) cells were monitored after controlled permeabilisation of the cells with digitonin, under conditions that preserve the exocytosis process. In order to further pinpoint functional domains in the toxin structure, and with the long-term aim of obtaining anti-bodies capable of antagonising each phase of the intoxication, antibodies were raised to isolated HC and LC.

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With respect to elucidating the toxin's molecular action, there is continued speculation that BoNT enzymatically inactivates its pharmacological target, like some other microbial toxins. As ADP-ribosylation of neuronal proteins were observed with impure preparations of type D (Ohashi et al., 1987; Matsuoka et al., 1987), a further search was carried out for such activity in homogeneous preparations of BoNT A and B towards nerve terminal components. In particular, a detailed comparison of the latter with type D was conducted, with special attention being devoted to ascertaining whether the supposed enzymic activity of BoNT D is related to its inhibition of transmitter release. In this context it also seemed relevant to establish if the ADP-ribosylated protein occurred in synaptic vesicles (where it could function in transmitter release) and whether it exhibited GTP-binding activity like the G-proteins covalently modified by pertussis or cholera toxins. The latter are of special interest because of novel G-proteins (eg. G_e) being implicated in exocytosis from other cells (Cockcroft et al., 1987). Finally, due to the definite involvement of phosphorylation/dephosphorylation of neuronal proteins in regulation of transmitter release (Llinas et al., 1985), possible effects of BoNT on the direct phosphorylation in vitro or on the phosphorylated state in situ of proteins in vesicles isolated from rat brain were evaluated.

EXPERIMENTAL PROTOCOLS
Purification of BoNT chains, fragments and antibodies to the latter.

BoNT A (strain NCTC 7272), B (Danish strain) and E (Alaskan strain) were purified as detailed previously (Tse et al., 1982; Evans et al., 1986; Schmidt and Siegel, 1986). Fully nicked preparations of the two latter toxins were obtained by treatment with trypsin (for B, 5 ug/ml at 22°C for 20 min; for E, 10 ug/ml at 37°C for 30 min) followed by the addition of excess trypsin inhibitor; in the case of BoNT B, the di-chain species was separated using FPLC on a Mono Q column (Maisey et al., 1988). HC and LC were purified from homogeneous preparations of BoNT A and B (as detailed in Maisey et al., 1988; Poulain et al., 1989) by adsorption onto a column of QAE-Sephadex, after treatment with 100 mM dithiothreitol and 2M urea, LC was removed in buffer containing 10 mM dithiothreitol and 2M urea. Following exhaustive washing of the gel with 0.1M NaCl in the latter medium, HC

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was then eluted by stepwise increase of NaCl concentration to
0.2M. The chains were renatured individually by removal of
dithiothreitol and urea upon dialysis at 4°C for >4h against
physiological buffers (see below). For reconstitution of the di-
chain form, equimolar amounts of HC and LC were dialyzed at 4°C
for 48-96h against 50 mM Tris-HCl, pH 7.7/50 mM NaCl. H_2L
fragment of BoNT A was prepared by proteolytic cleavage of the
latter with trypsin (0.05 mg/ml; 72h at 20°C) followed by FPLC on
a Mono Q column with salt elution of H_2L (detailed in Shone et
al., 1987). H_2 and LC were subsequently isolated from the
purified H_2L and refolded, as described above for the toxin's two
chains. All samples were stored at 4°C before use, any insoluble
material being removed by centrifugation prior to measurement of
protein content; toxicities of all the toxin chains and fragments
were determined by mouse bioassay (Williams et al., 1983). Their
purity was checked by SDS polyacrylamide gel electrophoresis,
performed under reducing and non-reducing conditions, followed by
Coomassie blue R250 staining; as a more stringent test for
contamination, the samples were monitored over several hours for
inhibition of neuromuscular transmission (see below). Polyclonal
antibodies to type A HC and LC were obtained from rabbits immuni-
zed repeatedly with the purified preparations. These were
assayed for (i) neutralisation activity by a back titration
toxicity assay in mice; (ii) titres against ^{125}I-BoNT using the
precipitation protocol detailed in Ashton et al., (1985) and
(iii) inter-chain cross-reactivitity by measuring the effective-
ness of toxin fragments/chains in preventing immuno-precipitation
of ^{125}I-BoNT.

Measurement of the effects of toxin preparations on transmitter release at motor endplates, Aplysia nerve terminals and brain synaptosomes.

Twitch tension was quantified at 23°C in mouse hemidiaphragm
following electrical stimulation (0.2 Hz square waves; 0.1 msec;
1.5 V) of the phrenic nerve, before and after bath application of
toxin samples; times taken to reduce muscle tension by 90% of the
initial value were measured in replicate tissues (Maisey et al.,
1988). Physiological Ringer oxygenated with 95% O_2/5% CO_2 was
used except when examining the ability of BoNT chains to antagon-
ise the inhibitory action of intact toxin; to minimise toxin

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uptake (Bandyopadhyay et al., 1987) in this case, the pre-incubation was carried out at 4°C in Ringer containing reduced Ca^{2+} (0.5 mM) and increased (5 mM) Mg^{2+} concentrations. Stock solutions of toxin preparations were dialyzed against the appropriate medium prior to dilution into the bath.

The buccal ganglion in Aplysia contain two large, identified cholinergic neurons afferent to the same post-synaptic cell, allowing electrophysiological measurement of ACh release from either; furthermore, inhibition of this release can be measured accurately after micro-injection of toxin into one of the pre-synaptic cell bodies or following bath application (Poulain et al., 1988). Likewise, it is possible to record release from non-cholinergic neurons in the cerebral ganglion. After impaling the neuronal somata (200-300 um diameter) with two glass micro-electrodes (3M KCl, 1-10 MΩ), the responses induced by a pre-synaptic action potential were recorded in the voltage clamped (at - 80mV) post-synaptic neurons. The amplitudes of these currents, which are proportional to the number of quanta released, are expressed as membrane conductance (nS) and in some illustrations are plotted as a percentage of the control response. The ganglia were perfused at 23°C with artificial sea water; after dialyzing toxin samples into the latter, they were bath applied or air-pressure injected (~1% of volume of the cell body) under visual (samples mixed [10%] with fast green dye) and electro-physiological monitoring by means of an additional microelectrode impaled into the pre-synaptic cell body. Such an unique system allowed internal administration of one toxin fragment or chain whilst another was bath applied at a chosen time interval. Effects of the toxin chains/fragments on Ca^{2+}-dependent resting and K^+-evoked release of ^3H-noradrenaline from rat brain synaptosomes were also assessed, using a standard assay (Ashton and Dolly, 1988).

Application of toxin samples to permeabilised PC-12 cells and quantitation of noradrenaline release.

PC-12 cells, grown in DMEM containing 10% (v/v) horse and 5% (v/v) calf serum, were plated on polylysine-coated multi-wells 1-2 days prior to use. Cells were loaded with ^3H-noradrenaline (1.6 uCi/ml; 15 Ci/mmol) for 1.5h at 37°C, washed and permeabili-sed for 5 min at 23°C (Peppers and Holz, 1986) with 7.5 uM

digitonin in KGEP buffer (139 mM K^+ glutamate, 20 mM Pipes, 1 mM Mg^{2+}-ATP, 5 mM EGTA, 5 mM glucose and 0.5 mM ascorbic acid, pH 6.6). Following removal of the medium, the quantities of radio-activity and lactate dehydrogenase (a cytoplasmic marker) released were determined. Cells were then treated for 20 min at 30°C with the appropriate toxin sample (dialyzed into KGEP) in KGEP containing 1 mM NAD^+ and 1 mg/ml albumin. Neurotransmitter release was elicited for 5 min at 23°C with 10 uM free Ca^{2+} by adding the appropriate amount (4.43 mM) of $CaCl_2$, calculated by a computer programme. Following scintillation counting of sample aliquots, Ca^{2+}-dependent release was quantified by subtraction of Ca^{2+}-independent efflux (measured similarly but in the absence of Ca^{2+}) from the total.

Assay of ADP-ribosylation and phosphorylation of synaptosomal and vesicular proteins in the presence of toxin.

In initial experiments, synaptosomes were employed so that BoNT-induced inhibition of transmitter release could be monitored (as detailed in Ashton and Dolly, 1988) under similar conditions being used for assaying enzymic covalent modification. Rat cerebrocortical synaptosomes were purified and incubated for 90 min at 37°C in the absence and presence of BoNT A, B or D (Wako, Tokyo) or pertussis toxin or cholera toxin. After preparing lysate of these synaptosomes, the samples were labelled for 2h at 30°C with 15 uM ^{32}P-NAD in buffer containing 10mM dithiothreitol plus type D (60 ug/ml) or activated pertussis toxin and subjected to SDS gradient gel electrophoresis/autoradiography (Ashton et al., 1988); two dimensional electrophoretic analysis was also performed. In a variation of this experiment, a lysate of untreated synaptosomes were labelled similarly with and without inclusion of BoNT A, B or D and, for comparison, activated pertussis or cholera toxin. Synaptic vesicles were isolated from rat cerebral cortex by density gradient centrifugation, as described by Huttner et al., (1983) but with the inclusion of protease inhibitors. Abilities of the various toxins to ADP-ribosylate vesicle proteins were assessed as noted above. Phosphorylation was also monitored in vesicles (Ashton et al., 1988a) by incubation at 37°C with ^{32}P-ATP in the absence and presence of BoNT, and Ca^{2+} plus calmodulin, with subsequent analysis of SDS gel electrophoresis/autoradiography. Addition-ally, phosphorylation of vesicular proteins by endogenous kinases

was examined, similarly, in vesicles purified from synaptosomes
that had been pre-intoxicated with BoNT. As a further variation,
intact synaptosomes were incubated at 37°C with ^{32}Pi for 90 min,
in the absence and presence of 200 nM BoNT; vesicles were then
isolated and electophoretic patterns of the phosphorylated
proteins compared. For investigations of vesicular G-proteins,
synaptic vesicles were separated from bovine brain, to a high
degree of purity, by differential centrifugation and gel filtra-
tion on a Sephacryl S-1000 column, according to the protocol of
Hell et al., (1988). Enrichment of synaptic vesicle was
quantified by ELISA of the marker, synaptophysin (p 38); assay of
Na^+ K^+ ATPase (ouabain-sensitive) and NADPH-cytochome C reductase
     indicated the degree of separation of plasma membranes and
endoplasmic reticulum, respectively. By means of a filtration
assay (Northup et al., 1982), total level of ^{35}S GTP S binding
could be determined whilst GTP binding to low M_r proteins was
measured, as detailed by Bhullar and Haslam (1987), after
electro-transfer of the proteins from SDS gels onto nitrocellu-
lose. As the classical, higher M_r G-proteins are inactivated by
the latter procedure, these were detected by immuno-blotting
using site-directed IgG antibodies to sequences of the a-subunits
of G_o and G_i, in conjunction with biotinylated anti-IgG and
streptavidin-peroxidase complex.

RESULTS AND THEIR INTERPRETATION
     The data obtained from the multi-disciplinary experimental
approaches can be comprehended most readily by dealing first with
the protein chemistry of the toxins, then the toxins' intracellu-
lar effects on transmitter release, followed by binding/uptake
and, finally, its molecular action.

1. PURITY AND BIOLOGICAL ACTIVITIES OF BoNT, FRAGMENTS AND
RENATURED OR RECONSTITUTED CHAINS

A prerequisite for all facets of the study is an adequate supply
of homogeneous BoNT, chains and fragments. As starting material,
pure BoNT A was isolated routinely in the complete di-chain form,
as established from the protein stained patterns of SDS electro-
phoresis gels run under reducing and non-reducing conditions
(Fig. 1A, tracks 4 & 6; 1E, tracks 1 & 6). In contrast, BoNT B
occurred predominantly in the single-chain form; thus, controlled

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trypsinisation followed by anion-exchange FPLC were required to yield the 'nicked' species (Fig. 1B; Maisey et al., 1988) which showed in increased level of toxicity in mice (2 x 10^8 LD_{50} units/mg) similar to that of type A (Table 1). This nicking procedure also raised the ability of BoNT B to block transmitter release when bath applied at the mouse neuromuscular junction and to rat brain synaptosomes (Table 1) or injected intra-neuronally into Aplysia neurons (Fig. 2B; Maisey et al., 1988), demonstrating that proteolytic processing (see later) is required for optimal activity. Notably, this di-chain preparation appears somewhat less potent than type A; such a small discrepancy could arise from several factors including slight differences in their structures, site of cleavage and, especially, because BoNT A and B apparently act at distinct sites in the release process (reviewed in Dolly, 1989). Type E was isolated exclusively in the single chain form but could be converted to the nicked di-chain species by trypsinisation (Fig. 1C), with a concomitant increase in toxicity but to a level lower than for BoNT A or B (Table 1) consistent with other findings (Sellin, 1987). Surprisingly, both forms of BoNT E were potent blockers of synaptosomal transmitter release; again, the precise reasons for this remain unclear. The activities of nicked and unnicked E preparations observed in Aplysia are presented later.

HC and LC from both toxin types were isolated chromato-graphically in electrophoretically pure state (Fig. 1A, tracks 1, 2; 1D, tracks 3 & 4). After refolding/renaturation during dialysis to remove the denaturants, the level of toxicity observed for the individual chains were ~5 orders of magnitude lower than native toxin (Table 1) and were found to be ineffective in other biological assays (see below), consistent with their high degree of purity. The constituent HC and LC of type A or B could be reconstituted to an appreciable extent into disulphide-linked di-chain species, as revealed by SDS electro-phoresis in the absence and presence of ß-mercaptoethanol (Fig. 1A, tracks 3,5; 1D, tracks 2, 5), with respective recoveries equalling 10% and 20% of the specific toxicity of the parent toxins (Table 1). Such incomplete reconstitution may have arisen from a proportion of the HC and LC refolding in a manner unfavourable for correct inter-chain disulphide bridge formation; obviously, this is essential for lethality in mice because mixing

the individually-renatured HC and LC failed to increase their toxicity or inhibitory action on evoked noradrenaline release from cerebrocortical synaptosomes (Table 1). In contrast to the constituent chains of either toxin, renaturation of mixtures of the chains from type A and B did not yield any detectable di-chain species when analyzed electrophoretically; accordingly, no increase in the peripheral toxicity was noticeable (Table 1). This reinforces the proposal (Maisey et al., 1988) that expression of the maximum level of toxicity in the whole animal requires HC and LC to be disulphide linked. Apparently, the association occurring between heterologous HC and LC, at least under the conditions evaluated herein, is incompatible with such covalent attachment, again, presumably due to some structural dissimilarities in the respective chains. H_2L was obtained by prolonged treatment of BoNT A with trypsin but a FPLC step was required to remove minor degradation products (Fig. 1E, tracks 4 & 5). Its purity was evident from the very low toxicity in mice (Table 1) and from the single protein band (M_r ~105kD) seen, under non-reducing conditions, in SDS electrophoresis gels; it was converted upon reduction to H_2 (M_r ~48kD) and LC (M_r ~52kD), the latter migrating identically to LC prepared from intact toxin (Fig. 1E). Also, the preparations of H_2 and LC isolated from H_2L each migrated as one major component when analyzed electro-phoretically (Fig. 1F). Following renaturation, the specific toxicities of H_2 (Table 1) and LC (~10^2 mouse LD_{50}/mg) isolated from H_2L were extremely low in keeping with a high level of purity; further examination of their biological activity is given below.

2. INTRACELLULAR ACTION OF BoNT, ITS CHAINS AND FRAGMENTS
(a) Direct demonstration that BoNT inhibits intracellularly Ca^{2+}-evoked release of ACh and other transmitters.

Micro-injection of nanomolar final concentrations of BoNT A (Fig. 2A) or B (Fig. 2B) into pre-synaptic cholinergic neurons in the buccal ganglion of Aplysia inhibited the evoked, quantal release of ACh (Poulain et al., 1988; Maisey et al., 1988). Analysis of recordings of the post-synaptic potentials showed that the observed blockade resulted from a reduction in the number of quanta released, their size remaining unchanged. BoNT

produced no detectable change in any of the other neurophysio-logical parameters measured, highlighting a specific inactivation of the release process. The toxin's effect was not restricted to cholinergic nerves; intracellular administration of similar concentrations into an identified, non-cholinergic neuron (its transmitter has not been identified unambiguously) in the cerebral ganglion of _Aplysia_ gave reduction of quantal transmitter release relative to a non-injected cell afferent to the same posysynaptic neuron (Fig. 2C). These experiments provide conclusive, direct evidence that BoNT acts intracellu-larly to block selectively the release of ACh and other trans-mitter(s), even when the ecto-acceptor binding and uptake steps are avoided. It must be emphasised that whilst ACh release could also be blocked by bath application of BoNT A (Fig. 2A), this treatment proved ineffective with the cerebral ganglion; there-fore, the nominal cholinergic specificity arises from the unique presence of an effective targetting/uptake system on these cells, as we deduced originally from autoradiographic studies on mammalian peripheral nerves (see Introduction). Further evidence for a broad specificity in the intracellular action of BoNT was gained from experiments on Ca^{2+}-induced noradrenaline release from cultured PC-12 cells. In the undifferentiated state, over-night treatment of this clonal line with 200nM BoNT A failed to affect K^+-stimulated, noradrenaline release (Fig. 3); this is presumably due to the toxin's inability to be internalized by the cells. Fortunately, toxin access could be achieved following digitonin permeabilisation under conditions that preserved the ability of the cells to perform Ca^{2+}-dependent exocytosis. In this experimental system, evoked release of ^{3}H-noradrenaline was diminished, though not abolished by BoNT A (Fig. 3); specificity of the effect was established by an observed abolition of the inhibition when anti-BoNT antibodies were included together with the toxin. Based on these and other recent investigations on chromaffin cells (Penner et al., 1986; Bittner et al., 1989), it can be concluded that BoNT inactivates an ubiquitous, intracellu-lar component concerned with the Ca^{2+}-dependent release process, though other more diverse systems should be tested.

(b) <u>HC</u> <u>and</u> <u>apparently</u> <u>domain(s)</u> <u>in</u> <u>the</u> H_1 <u>moiety</u> <u>of</u> <u>HC</u> <u>mediate</u>
<u>the</u> <u>intra-neuronal</u> <u>blockade</u> <u>of</u> <u>ACh</u> <u>release</u> <u>in</u> Aplysia: <u>comparison</u>
<u>with</u> <u>permeabilised</u> <u>cells.</u>

To pinpoint regions in the BoNT structure that contribute
to its intracellular action, quantal release of ACh was
monitored electrophysiologically in <u>Aplysia</u> neurons before and
after micro-injection of individually-renatured chains, fragments
of the toxin or mixtures thereof. HC or LC of BoNT B (Fig. 4,
upper panels) proved ineffective over several hours when placed
alone inside the cells, as did the separate chains of type A
(Fig. 5, middle panel, Fig. 6D). However, administration into
the pre-synaptic cell body ofa low concentration (1-2.5nM final
inside the cell) of an equimolar mixture of HC and LC of either
toxin type (Fig. 4, lower panel, Fig. 5A) led to a blockade
of ACh release. This not only demonstrates that both chains were
required but also excludes a need for the inter-disulphide bond,
since the reducing intracellular environment should prevent re-
oxidation. Being somewhat surprised at the need for the HC in
addition to LC, the H_2L_A fragment (lacking H_1 moiety) was tested
and found to be inactive (Fig. 6E); the presence of an inter-
chain disulphide bond could not be deemed responsible for this
lack of an effect because a mixture of H_2 and LC, likewise,
showed no inhibition (Poulain <u>et</u> <u>al.,</u> 1989). Because LC derived
from the H_2L preparation proved active when HC was added (Fig.
7B), (consistent with results of reconstitution experiments; see
above and Table 1), and H_2L plus HC were effective (Poulain <u>et</u>
<u>al.,</u> 1989; Fig. 7A discussed later), these collective findings
point to a requirement for LC **plus** H_1 moiety. Further tests
involving H_1 (its isolation in the native state is currently
being developed) are needed to document this proposal. In the
meantime, it has gained further support from experiments on a
preparation composed exclusively of single-chain BoNT E (Fig. 1C)
that was devoid of inhibitory action intracellularly (Fig. 6C)
even at high concentrations (50nM) but which became fully active
after nicking (Fig. 6A,B). The intriguing question this raised
of whether nicking induces an active state of LC and/or HC was
then addressed. Pre-injection of a neuron with H_2L before
bath applying unnicked E gave no alteration in release (Fig. 6E),
leading to the suspicion that H_1 was defective. Such a notion
seems correct because of the observation that injecting HC_A into

the cholinergic cell enabled the single-chain E to block release
(Fig. 6D); also, this exemplifies how heterologous toxin types
can function together (see p. 14). These novel results reinforce
the requirement for H_1 (together with LC) and unveil the
additional need for activation of H_1 during nicking (via a
conformational change or by direct processing), since the nicked
toxin E was fully active. The latter possibility could accord
with reports (DasGupta and Sugiyama, 1972) that removal of only a
few amino acids from the C-terminus of the single-chain form
(though further cleavage in the whole animal was not excluded)
resulted in the expression of the toxin's full toxicity in mice.
Involvement of the alternative (ie. cleavage between HC and LC)
could be ascertained in our system by showing if single-chain
BoNT E only becomes active when processed at the C-terminus,
provided that Aplysia neurons lack proteases capable of creatina
the di-chain species.

In attempts to establish whether the two identified domains
(LC and H_1) were essential for the toxin's action in mammalian-
derived cells, effects of HC and LC on exocytosis from permeabi-
lised PC-12 cells were examined. Whereas HC_A had minimal effect
even at high concentrations (up to 200nM), LC_A alone produced a
large reduction in the extent of Ca^{2+}-evoked release of ^{3}H-
noradrenaline from the digitonin-treated cells, with a potency
similar to that of native BoNT (Table 2). It remains to be
determined,by performing further dose-response curves, if the
level of inhibition seen in PC-12 cells with a series of sub-
maximal concentrations of LC can be altered by the presence of
HC. Furthermore, documentation is needed to establish the
similarity of catecholamine release from such undifferentiated
PC-12 cells to the quantal release of ACh measured herein from
neurons. Already, it has emerged that increasing the intra-
cellular Ca^{2+} concentrations to 100uM is unable to reverse BoNT
A-induced inhibition of release from the permeabilised cells, in
direct contrast to the reversibility observed upon raising Ca^{2+}
concentration at both motor nerve terminals or brain synaptosomes
(reviewed in Sellin, 1987; Sanchez-Prieto et al., 1987).
Although an invertebrate neuronal source was employed in the
studies herein, these cells exhibit no major differences relative
to transmitter release from mammalian nerve terminals. Apart
from possible minor but yet important variations in the release

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process (or its control) in these widely different models, the harsh treatment of cells with detergent during permeabilisation could lead to a loss of some factors that would, otherwise, necessitate the presence of HC to facilitate the action of LC. Such numerous possibilities can only be distinguished by additional investigations; towards this end, recent reports have appeared on the blockade by LC of BoNT or tetanus toxin of catecholamine release from permeabilised adrenal medulla chromaffin cells (Bittner et al., 1989; Ahnert-Hilger et al., 1989) but, again, differences may exist between these exocrine cells and nerve endings.

Returning briefly to the experiments on Aplysia, it is pertinent to consider the relevance of our findings to the question of whether HC and LC associate together (non-covalently) inside the neuron thereby creating an active species, or if they act in concert (via different components or distinct sites on a single macromolecule). The ability of HC_A (noted above) to make unnicked BoNT E (Fig. 6D) or H_2L_A (Fig. 7A) assume inhibitory activity suggests that intimate association between the chains cannot occur. Even if the di-chain form of E was produced by postulated proteases in the cells, and further assuming that the inter-chain disulphides in E and H_2L become reduced, their constituent polypeptides would nevertheless be unlikely to dissociate in the cellular milieu (2M urea and 10-100mM dithiothreitol is required to separate them in vitro; see p.3). Hence, an attractive suggestion, consistent with all available data, is that the toxin's target in permeabilised cells exists (naturally or following alteration as a result of experimental manipulation) in a state susceptible to LC alone whilst in neurons (at least in Aplysia) HC mediates conversion of this substrate to a form vulnerable to LC. Obviously, much further research is needed to evaluate this postulated scheme but that could give much insight into components underlying and/or controlling release in different cell types.

3. NEURONAL BINDING AND UPTAKE OF BoNT: STRUCTURAL REQUIREMENTS IN Aplysia NEURONS AND MAMMALIAN NERVE TERMINALS

In view of (i) the preferential BoNT susceptibility of peripheral cholinergic nerves; (ii) the unique occurrence therein

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of acceptor-mediated toxin uptake (see Introduction) and (iii)
blockade of release when BoNT was bath-applied to cholinergic but
not non-cholinergic neurons of _Aplysia_ ganglia (see section 2),
identification was sought for regions of the toxin responsible
for interaction with neuronal ecto-acceptors and the subsequent
internalization. Although such experiments were readily feasible
in _Aplysia_ cholinergic neurons, where possible comparisons were
made with murine motor nerves. The renatured HC_A was found to
become internalized when bath-applied because ACh release
decreased from a neuron in the ganglion that had been injected
with LC_A (Fig. 5B, 7B). In contrast, uptake of LC_A did not occur
as reflected by the lack of change in release even when high
concentrations (100 nM) were added to the solution bathing the
tissue, after internal administration of HC_A (Fig. 7C). Thus, HC
alone or as part of the intact toxin can be taken up into the
neuron in an active form; additionally, it can mediate the
internalization of LC because bath application of HC_B and LC_B in
sequence resulted in blockade of neurotransmitter (Fig. 8A).
This uptake involves tight interaction of HC with the membrane
since removal of unbound chain by extensive washing (75 min) did
not prevent its ability to internalize LC_B when added later,
resulting in a decrease of transmission (Fig. 8B). As expected,
in the reverse experiment, pre-equilibration with LC_A (up to
100nM) in the bath followed by its removal and subsequent addi-
tion of HC_A produced no change (Fig. 8D). Interestingly, HC_B
could also internalize LC of type A and reduce the measured post-
synaptic response (Fig. 8C), re-emphasising the ability of chains
from different toxin types to work in concert (cf. Fig. 6D).
Moreover, the heterologous chains of types A and B again worked
effectively when HC_A was applied externally and LC_B injected
(Fig. 5C); in view of the fact that this latter mixture of chains
failed to form a disulphide-linked complex (see p. 9), it can be
concluded that uptake of non-covalently associated chains occurs
in _Aplysia_.

When related experiments were carried out in the nerve-
diaphragm twitch preparation, somewhat different results emerged.
Although addition to the bath of HC_A and LC_A gave a blockade of
neuromuscular transmission, the mixture of chains was very much
less potent than BoNT with 30nM of the separated chains being
equivalent to 0.1nM intact toxin (Fig. 9A). Likewise, 30nM

HC exhibited little or negligible ability to antagonise the
neuroparalytic effect of a relatively low concentration (0.3nM)
of BoNT (Fig. 9B), even though conditions were employed (4°C,
lowered Ca^{2+} and increased Mg^{2+} concentrations) in the pre-
equilibration with HC to minimise its internalization before
addition of BoNT. A simple interpretation of these findings is
that only the di-chain form of toxin is efficiently internalized
at mammalian motor nerve endings (Maisey et al., 1988). This is
based on the reasonable assumption that during the course of the
experiments reformation of disulphide-linked species occurs from
a small proportion of HC and LC; indeed, the elevation in levels
of toxicity seen when reconstituting the chains (Table 1) was
always associated with a concomitant creation of the di-chain
form (Fig. 1A,D).

Due to the less stringent structural requirements for toxin
internalization in Aplysia cells, these could be exploited in
pinpointing sections of HC concerned with uptake. H_2L alone can
traverse the membrane as shown by the reduction in ACh release
recorded when it was bath applied and HC injected (Fig. 10A).
The absence of an effect with H_2L until HC was added to the
medium reaffirms the requirement for H_1 moiety [discussed in
(2b)]; this applied whether H_2L was administered externally (Fig.
10B) or internally (Fig. 7A). As LC alone was shown earlier not
to be taken up (Fig. 7C, 8D), H_2 must be responsible for the
uptake of H_2L. In fact, isolated H_2 is shown directly to promote
internalization of LC, release being blocked in a neuron pre-
injected with HC; H_2 proved ineffective prior to addition of LC
(Fig. 11B) and vice versa (Fig. 11A); lack of change in release
from either control neuron (not injected with HC) excludes any
contribution from contaminations (with native toxin, H_2L or HC)
in bath-applied LC or H_2 samples. Thus, to our knowledge, direct
and conclusive evidence is provided for the first time that H_2
domain of HC can mediate uptake at least in Aplysia; it is
now warranted to examine how this relates to known pore-forming
activity (see Introduction). Interestingly, the potency of the
toxin chain/fragments in Aplysia approximates to that of the
intact molecule, in direct contrast to the situation (noted
above) at the mouse endplates. Binding of toxin to acceptors in
vertebrate neuronal membranes seems to involve the H_1 region of
HC since after its removal H_2L is unable to bind synaptic

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membranes (Shone et al., 1985). This information together with
the ineffectiveness of H_2L on the nerve-muscle twitch preparation
(Table 1) and the efficient uptake by the latter of di-chain
species only lead to the speculation that mammalian nerve endings
have evolved an exquisite sensitivity to BoNT because domains in
H_1 together with H_2 and, apparently, structures in whole molecule
contribute to the high affinity binding that results in the
uptake necessary for intoxication. On the other hand, the
primordial acceptors in Aplysia neurons exhibit less extensive
structural requirements for that interaction with toxin (predomi-
nantly via H_2) and, hence, display an apparent lower affinity;
of course, efficiency of the actual uptake mechanism could also
be a contributory factor to the subtle differences emerging
between the systems.

4. INVESTIGATIONS OF THE MOLECULAR ACTION OF BoNT

(a) Ability of impure type D but not homogeneous BoNT A or B
to ADP-ribosylate synaptosomal proteins: relevance to their
inhibition of transmitter release

As the procedures used in our early experiments failed to
reveal enzyme activity in BoNT A or B, further investigations
were carried out using the latter and, for comparison, commercial
preparations of type D, pertussis and cholera toxins. The two
latter toxins caused ADP-ribosylation of their respective G-
proteins (G_i and G_o) and, in addition, cholera toxin labelled
some larger proteins (Fig. 12A, tracks 4 & 5). However, lysates
of cerebrocortical synaptosomes (Fig. 12A, tracks 2 & 3) could
not, as before, be labelled with 200nM BoNT A or B following
incubation at 20-37°C for up to 3h with ^{32}P-NAD (various
concentrations) in the absence or presence of additives required
for maximal ADP-ribosyltransferase activity in certain toxins
(eg. 10mM dithiothreitol, 4mM $MgCl_2$, 4mM $CaCl_2$ or 100uM GTP S).
Furthermore, renatured HC_A and LC_A, alone or together (30nM),
were ineffective under all conditions tested. In direct con-
trast, similar treatment of synaptosomal lysate with type D
toxin (60 ug/ml) caused ADP-ribosylation of 24,000 M_r protein
with (Fig. 12A, track 1) or without inclusion of reducing agent;
whereas Mg^{2+} increased the amount of labelling, it remained
unaltered when Ca^{2+} or GTP S was added. Phosphodiesterase

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ti...tment removed much of labelling produced by D toxin, consistent with the presence of ADP-ribose moiety. On two-dimensional gel analysis (isoelectric focussing in urea followed by SDS electrophoresis), the labelled component was resolved into 3 bands with pI values of 5.5, 5.1 and 4.8; the higher resolution of this system showed that the most acidic protein has a slightly higher M_r (~25,000) than the other two. To evaluate if this covalent modification occurred in intact nerve terminals and, thus, help ascertain whether it is relevant to intoxication, synaptosomes were pre-incubated with each toxin in the absence of radiolabel; lysates were then prepared and a back titration performed using ^{32}P-NAD and BoNT D (Ashton et al., 1988). Pre-treatment with BoNT A, B or D failed to alter the extent of subsequent ADP-ribosylation by D toxin relative to the control (Fig. 12B, tracks 1 to 4). In a similar study,a pre-incubation of synaptosomes with pertussis (Fig. 1B, tracks 5 & 6) or cholera toxins abolished the labelling normally seen with the respective toxin, as expected. Hence, none of the BoNT samples were causing measurable ADP-ribosylation in the intact synaptosomes nor did they affect labelling of lysate by type D. Yet, treatment of synaptosomes under the same conditions with BoNT A, B or D inhibited Ca^{2+}-dependent K^+-evoked release of ^{3}H-noradrenaline (Fig. 13) without affecting the Ca^{2+}-insensitive efflux of radioactivity or synaptosomal integrity, as judged by unaltered uptake of transmitter. Thus, it can be deduced that the ADP-ribosylation seen with toxin D is unrelated to its blockade of transmitter release and probably is mediated by contaminating C_3 (an ADP-ribosyltransferase not specific for neurons) as first demonstrated by Rösener et al. (1987) in another cell type. Obviously, the neurotoxin in the D preparation must have entered the nerve terminals and inhibited transmitter release whilst the contaminant appears to have been excluded and, thus, ADP-ribosylation prevented.

Notwithstanding this discrepancy, it is noteworthy that the 24,000 M_r protein modified by the D toxin could be demonstrated in purified synaptic vesicles, together with the substrate for pertussis toxin (Fig. 12C, tracks 1 & 4); again, BoNT A or B were unable to exhibit protein labelling in this concentrated vesicle suspension (Fig. 12C, tracks 2 & 3). In view of G-proteins being implicated in secretion (see Introduction), the protein labelled

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by D toxin in vesicles was examined for GTP-binding activity because of its possible relevance to transmitter release.

(b) <u>Demonstration of low molecular weight GTP-binding proteins in synaptic vesicles</u>

Following large-scale purification of vesicles from bovine brain they were enriched in the vesicle marker p38 (synapto-physin) as quantified by ELISA (Matsuoka and Dolly, 1990; [submitted]) or immuno-blotting (Fig. 14A). When nitrocellulose blots of SDS gel of the purified vesicles were incubated with a-^{32}P-GTP, three low M_r bands (27,000 major, 26,000 and 24,000 minor) were observed (Fig. 14B) whilst the classical GTP-binding proteins (G_o, G_i) occurred predominantly in the plasma membrane. These low M_r G-proteins were clearly distinct from ras p21 proteins, another family of low M_r (21,000) G-proteins which was revealed, by immunoblotting, in a different fraction (containing plasma membrane) of the gel permeation profile (Fig. 14C). It is of special interest that these novel, low M_r G-proteins are of similar size to the substrates labelled by type D toxin, parti-cularly because these have been identified as rho gene products (Yamamoto <u>et al.</u> 1988; Quilliam <u>et al.</u> 1989). Investigations on yeast mutants have identified genes coding for low molecular mass GTP-binding proteins involved in constitutive secretory pathway (Burgoyne, 1988). Similar sized G-proteins have also been detected on chromaffin granules (Burgoyne and Morgan, 1989) concerned with catecholamine secretion; in fact, guanine nucleo-tides were found to modulate Ca^{2+}-dependent catecholamine release from permeabilised chromaffin cells (Bittner <u>et al.</u> 1986). Based on these collective findings, it is tempting to speculate that the small G-proteins studied herein may be concerned with exocy-tosis, though their implication in the action of BoNT remains to be established.

(c) <u>BoNT does not affect phosphorylation of proteins in synaptic vesicles.</u>

Because of the apparent importance of phosphorylation/ dephosphorylation in transmitter release (see Introduction) and the report that BoNT alters phosphorylation in <u>Torpedo</u> electroplax preparations (Guitart <u>et al.</u> 1987), this possibility

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was examined further despite our previous observations that the toxin did not alter such covalent modification (Dolly et al. 1987). In this study, attention was devoted to synaptic vesicles; since they represent a minute fraction of total synaptosomal protein their modification by BoNT could have escaped detection.

Synaptic vesicles were fractionated from rat cortex synaptosomes before and after K^+ depolarization, with and without BoNT A pre-treatment that virtually abolishes Ca^{2+}-dependent release of transmitters (Ashton and Dolly, 1988). Electro-phoretic monitoring of basal phosphorylation, and after activation with Ca^{2+}-calmodulin, showed no toxin-induced change in the resultant patterns (Fig. 15A). Furthermore, vesicles isolated from non-toxin treated tissue was incubated with BoNT A during phosphorylation reaction with ^{32}P-ATP but this, also, failed to detect any alteration. In view of the possibility that any change in vesicular pattern due to the toxin might be reversed during the subsequent separation of vesicles, labelling with ^{32}Pi of untreated and intoxicated synaptosomes was carried out, with inclusion of a cocktail of kinase and phosphatase inhibitors during the fractionation. This procedure, likewise, was unable to detect any difference attributable to BoNT in the phosphorylation patterns of vesicles prepared from either polarised or depolarised synaptosomes (Fig. 15B).

CONCLUSIONS

In the first 18-months of this contract, significant advances were accomplished encompassing 2 of the 4 main objectives. These include:
(i) Preparation/complete purification and characterization of the biological activities of BoNT A, B and E (with and without nicking/processing), HC and LC of BoNT A and B, H_2L_A and H_2; isolation of H_1 is nearing completion.
(ii) Conclusive demonstration of an intracellular target for BoNT in invertebrate neurons and PC-12 cells whilst in the case of mammalian motor nerve endings and brain synaptosomes strong evidence (albeit indirect) was, also, obtained for an intra-neuronal action.
(iii) Establishing the widespread importance of the toxin's target by showing that BoNT can block Ca^{2+}-dependent secretion in a number of cell types.

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(iv) The nominal cholinergic specificity of BoNT in vivo being ascribed to the unique presence of toxin ecto-acceptors on these neurons that afford targetting/uptake.

(v) LC alone proved effective in inhibiting exocytosis from permeabilised PC-12 cells whereas, additionally, H_1 was required for its intracellular blockade of ACh release from Aplysia neurons.

(vi) Toxin binding and uptake in Aplysia neurons was seen to be mediated primarily by H_2 fragment, though the more efficient internalization observed indirectly at motor nerve terminals needed additional structural features present in the di-chain native toxin.

(vii) Extensive investigations on the molecular action showed that BoNT A, B or D in brain synaptosomes does not induce ADP-ribosylation; likewise, BoNT A was found not to alter phosphorylation.

(viii) GTP-binding proteins were detected in synaptic vesicles and these had M_r values identical to the band ADP-ribosylated by an impure preparation of type D botulinum toxin.

The main implications of these findings for future research are:

(a) Having identified domains in the toxin molecule that are responsible for binding/uptake (H_2) and intracellular action (LC and H_1) in Aplysia neurons (a model amenable to such investigations), it is imperative to ascertain if these assignments are correct for mammalian nerve cells. To achieve this, methods are being developed (eg. liposomes and axonal transport) that will allow toxin/fragments/chains to be delivered inside motor neurons, the prime target. Additionally, encouraging attempts have already been made to use rat pituitary cells together with patch pipette techniques in this study; this system has facilitated intracellular administration of protein samples whilst recording exocytosis by means of quantifying membrane capacitance changes (Mason et al., 1988). Such sophisticated approaches avoid the damage caused to cells by detergent permeabilisation and would, therefore, test the validity of results obtained with BoNT on PC-12 or chromaffin cells, as well as establishing if BoNT truly affects hormone secretion.

(b) Notwithstanding the need for additional structure/activity studies in a diversity of cell systems, production of antibodies

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to the functional *regions* already identified and isolated (H_2, H_1, HC and LC) is a priority because this could provide effective neutralization agents and aid the further identification of essential domains.

(c) As motor nerve endings require the disulphide-linked di-chain form of BoNT for efficient action, future studies on targetting agents to this site will be directed to keeping the HC intact and altering LC to remove its ability to affect secretion; this can now be attempted realistically using protein chemistry as well as molecular genetics because the gene for BoNT A has been cloned in the laboratory of our collaborator Prof. J. Melling.

(d) Higher resolving methods must be applied for monitoring the toxin's molecular action; to date, two dimensional electro-phoretic analysis following chase labelling with high specific activity ^{35}S-methionine seems promising. Use of an alternative strategy, in which a variety of drugs were tested for their ability to antagonise the action of BoNT, is implicating an interaction with the cytoskeleton; this novel and important observation is being pursued intensively.

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Table 1. Biological activities of BoNT preparations, fragments and renatured or reconstituted chains.

	Mouse bioassay[a] (LD_{50}/mg protein)	Inhibition of neuromuscular transmission (time [min] for 1nM to give a 90% reduction of twitch tension)	K^+-evoked release[b] noradrenaline from synaptosomes in the presence of 50nM of each protein (% of control)
BoNT A	2×10^8	100	30
BoNT B	2×10^7	240	87
BoNT B (nicked)	2×10^8	140	63
BoNT E	2×10^5	n.d.	28
BoNT E (nicked)	1×10^7	n.d.	25
H_2L_A	$<10^2$	60nM ineffective	100
H_2A	$<10^2$	–	
LC_A derived from H_2L	10^2	–	102
LC_A	10^2	30nM ineffective over 6h	95
HC_A	$>2 \times 10^3$	" "	101
LC_B	10^3	n.d.	97
HC_B	$<5 \times 10^3$	n.d.	95
Chains renatured together:			
$LC_A HC_A$	2×10^7	see text	60
LC_A (from H_2L) HC_A	10^7		
$LC_B HC_A$	10^3	n.d.	101
$HC_B LC_B$	4×10^7	n.d.	n.d.
Mixture of renatured chains:			
$HC_B + LC_B$	10^3	see text	95
$HC_A + LC_A$	10^3	" "	92
$HC_A + LC_B$	10^3	" "	106

[a] Mouse toxicity results were obtained with not less than three different preparations of each protein species.

[b] Synaptosomes were incubated for 90 min at 37°C with 100nM [^{3}H]noradrenaline in the presence of each toxin sample. Results are the mean for six determinations; the range was not greater than ± 8% of the values quoted.

Table 2.   Inhibition of Ca^{2+}-elicited [^{3}H]noradrenaline release from
          digitonin-permeabilised PC-12 cells by BoNT A or its chains

Treatment	Release (% of control)
0.75nM BoNT A (200nM)	29.3 (31.1; n=12)
0.75nM LC_A	27.3
100nM HC_A	87.5 (98.1; n=12)
0.75nM LC_A plus 100nM HC_A	19.2

Average values are shown for Ca^{2+} (10μM free)-dependent release measured
as outlined in Fig. 3; Ca^{2+}-independent release has been subtracted.
Preparations of BoNT and its chains were dialyzed into KGEP prior to
use.   In control experiments, the buffers used to prepare the various
toxin samples were treated similarly to the BoNT preparations (including
dialysis) and shown to have no detectable effects on [^{3}H]noradrenaline
release.

Figure Legends

Fig. 1. SDS polyacrylamide gel electrophoresis of purified BoNT A,
B and E, fragments and their chains before and after reconstitution
     Gradient or 10% acrylamide gels were run in the absence and
presence of ß-mercaptoethanol and protein bands stained with
Coomassie blue.
A. Electrophoresis was carried out using 10% acrylamide gels
under reducing (tracks 1-4) and non-reducing (5, 6) conditions.
Track 1, LC of BoNT A; 2, HC of BoNT A; 3 & 5, equimolar mixture
of LC and HC of BoNT A renatured together by extensive dialysis
from urea- and dithiothreitol-containing buffer into non-
denaturing medium; 4 & 6, BoNT A.
B. Gradient (4-30% w/v) gel electrophoresis was carried out under
reducing conditions. Tracks 1, molecular mass (kDa) markers
(phosphorylase b. 94; serum albumin, 67; ovalbumin, 43 ; carbonic
anhydrase, 30;soybean trypsin inhibitor, 20 and a-lactalbumin,
14.4; 2, BoNT B that was trypsin-treated (5 ug/ml final concen-
tration, 30 min at 22°C) and subjected to FPLC anion-exchange
chromatography; 3, native, untreated BoNT B and 4, BoNT B,
trypsin-treated as in track 2, prior to FPLC.
A. and B. are from Maisey et al. (1988).
C. Electrophoresis was carried out as in (A) under reducing
(tracks 1-3) or non-reducing (tracks 4 & 5) conditions. Tracks 1
& 4, unprocessed native BoNT E; 2 & 5, BoNT E following
treatment with trypsin (10 ug/ml for 30 min at 37°C); proteolysis
was arrested by the addition of soybean trypsin inhibitor (25
ug/ml); 3, molecular weight markers as in (B). Note that under
reducing conditions the presence of the toxin's constituent
chains indicates that proteolysis has converted the single-chain
to the nicked di-chain form. From Poulain et al. (1990); J.
Biol. Chem., in press).
D. Gels run as in (A) under non-reducing (tracks 1 & 2) and
reducing (tracks 3-6) conditions. Tracks 1, molecular mass
standards as in (B); 2, equimolar mixture of LC_B and HC_B renatured
together by dialysis from buffer containing dithiothreitol and
urea into buffer devoid of denaturants; 3, HC_B; 4, LC_B;
5, reconstituted HC and LC as in track 2; 6, standard markers as
in (B). Adapted from Maisey et al. (1988).
E. Gradient gel (5-20% w/v acrylamide) electrophoresis was
performed under reducing (tracks 1-3) and non-reducing (tracks
4-6) conditions. Tracks 1 & 6, BoNT A; 2 & 4, H_2L after
purification by FPLC; 3, molecular mass (kDa) markers; thyro-
globulin subunit, 330; ferritin, 220) plus standards as in (B);
5, BoNT A after trypsin treatment for 72h.
F. Electrophoresis was carried outusing a 10% (w/v) acrylamide
gel run under reducing conditions. Track 1, LC prepared from
H_2L; 2, molecular mass markers (phosphorylase b, serum albumin,
ovalbumin and carbonic anhydrase); 3, H_2 prepared from H_2L.
E. and F. are from Poulain et al. (1989).

Fig. 2. Action of BoNT A and B on transmitter release from
ganglionic neurons of Aplysia
A. Inhibition of ACh release by extra- or intra-cellularly
applied BoNT A. Transmitter release was evoked at an identified
cholinergic synapse in the buccal ganglion of Aplysia by a 3-sec
depolarization (to + 10 mV) of the presynaptic neuron under
voltage clamp. The amplitudes of long duration inhibitory
postsynaptic current recorded at 23°C in the voltage-clamped (-80
mV) postsynaptic neurons are expressed as conductance (nS).
After control recordings, BoNT type A was bath-applied at 7nM

(hatched bar,▲) or, in another preparation, air-pressure
injected into the cell body of the presynaptic neuron (arrow, ●)
to give a calculated intracellular concentration of ~5nM.
(Insets) Postsynaptic recordings at the point indicated in the
control conditions (left) and during the decrease in ACh
release (right) resulting from the intraneuronal administration
of BoNT. (Vertical bar = 300 nS; horizontal bar = 1.2 sec.).
In the reported experiments the calculated miniature postsynaptic
current (MPSC) amplitudes were 0.99±0.23 nS for control and
1.05±0.17 nS after 3h of bath application of BoNT and 1.55±0.20
nS for control and 1.62±0.25 nS 3h after injection of BoNT. Each
value of MPSC was calculated from at least five subsequent
recordings. All parts of the experiments have been repeated on
at least three separate preparations, and the same pattern of
blockade with BoNT was observed. From Poulain et al. (1988).
B. Decrease in ACh release induced by intracellular application
of native and 'nicked' BoNT B. In the buccal ganglion of
Aplysia, ACh release was evoked by a pre-synaptic action
potential. Amplitude of the postsynaptic response was plotted
against time as a percentage of the control response, before and
after intracellular injection (arrow) of BoNT B. Similar amounts
(~1% of the cell body volume) of unnicked BoNT B (▲, 8uM) or of
the two-chain, 'nicked' form of BoNT B (●, 8uM; O, 0.8uM) were
injected into the presynaptic cell. 'Nicking' the toxin
increased its potency with the 'unnicked' material producing a
blockade similar to that seen with a 10-fold lower concentration
of the 'nicked' preparation. From Maisey et al. (1988).
C. Inhibition of transmitter release in a non-cholinergic neuron
induced by intracellular injection of BoNT A. Transmitter release
was evoked at a non-cholinergic synapse in the cerebral ganglion
of Aplysia by a pre-synaptic action potential; the resultant
postsynaptic responses were recorded as membrane potentials. In
contrast to the buccal ganglion, repetitive activation of this
synapse modifies its efficacy slightly. Consequently, the
changes (%) in amplitude of the postsynaptic response (●) after
an injection of BoNT type A (arrow) into the pre-synaptic neuron
were compared with the responses (O) of non-injected equivalent
neuron, afferent to the same postsynaptic cell and releasing the
same neurotransmitter (a schematic drawing of the synaptic
connections is shown). The control neuron and the injected one
were stimulated alternately. Calibration: 100% = 1.8mV (●) or
1.5mV (O). From Poulain et al. (1988).

Fig. 3. The effect of BoNT A on neurotransmitter release from
non-permeabilised and permeabilised PC12 cells
A. Intact cells were incubated at 37°C overnight in 250ul of
medium without (1, 2,3) and with (4,5) 200nM BoNT A. This was
replaced with 250uL of medium containing 0.4 uCi [7,8-^{3}H]nor-
adrenaline (15 Ci/mmol) for 1.5h at 37°C. After three washes
with medium containing 25mM HEPES, pH 7.4, intact cells were
incubated at room temperature for 5 min without any additions
(1,4); with 45mM KCl (2,5); with 45mM KCl and 10mM EGTA (3).
B. Permeabilised cells were treated for 20 min at 30°C with
KGEP/albumin/NAD$^+$ without (i,ii) and with (iii,iv) 200nM BoNT A,
followed by a 5 min incubation with KGEP/albumin without (i, iii)
and with (ii, iv) 10uM free Ca^{2+}. The amount of radioactivity
released was quantified by scintillation counting of aliquots and
expressed as % of the cellular content (in the case of permea-
bilised cells after digitonin and toxin treatment). Points
(± SD) presented are from 4 independent wells for each condition
and are representative of at least 10 experiments.

Fig. 4. <u>Both</u> <u>chains</u> <u>of</u> <u>BoNT</u> <u>B</u> <u>are</u> <u>required</u> <u>intracellularly</u> <u>to</u> <u>block</u> <u>transmitter</u> <u>release</u> <u>in</u> Aplysia <u>neurons.</u>
    ACh release was evoked by a presynaptic action potential; responses were recorded in the voltage-clamped postsynaptic neuron and expressed as membrane conductance (nS). Intracellular application (arrow) of individually renatured LC(a)or HC(b) of BoNT B failed to produce a blockade; however, when injected together (c) a decrease in release was observed. From Dolly <u>et</u> <u>al.,</u> (1988).

Fig. 5. <u>Contributions</u> <u>of</u> <u>HC</u> <u>and</u> <u>LC</u> <u>to</u> <u>the</u> <u>intoxication</u> <u>process</u>
    These experiments were performed on the buccal ganglion of <u>Aplysia</u> and ACh release was evoked by a presynaptic action potential; in all cases, the postsynaptic responses recorded in a control neuron afferent to the injected cell [insert in (b) shows the synaptic connections] remained unchanged.
(A) After control measurements were made, equimolar concentrations of separately renatured LC and HC of BoNT A were mixed and injected (↑) into the presynaptic neuron; this resulted in a reduction of relase. The insert represents an actual response elicited by an action potential at a time indicated (●) before (left) and after (right) the injection. Vertical calibration is 80mV for the pre-synaptic action potential and 1000 nS for the postsynaptic response; the horizontal calibration is 75 ms.
(B) Intracellular administration (↓) of LC of BoNT A was ineffective but release was decreased following addition of 20nM HC (hatched area) to the extracellular medium.
(C) Bath application of 20nM HC of type A (hatched area) did not alter secretion; after intracellular injection (↓) of LC of BoNT B, a dramatic reduction in release ensued, showing that the chimeric toxin is active. (Modified from Poulain <u>et</u> <u>al.,</u> 1988).

Fig. 6. <u>Effect</u> <u>of</u> <u>nicked</u> <u>and</u> <u>unnicked</u> <u>BoNT</u> <u>E</u> <u>on</u> <u>ACh</u> <u>release</u> <u>in</u> Aplysia <u>in</u> <u>the</u> <u>absence</u> <u>and</u> <u>presence</u> <u>of</u> <u>$HC_A$and</u> <u>H_2L_A</u>
    Postsynaptic responses (%) were recorded at 22°C in neurons of the buccal ganglion.
A. Bath application of 10nM nicked BoNT E (horizontal lines).
B. Following equilibration, nicked BoNT E was administered into the cell body of a presynaptic neuron (arrow) to an approximate intracellular concentration of 10nM.
C. Following equilibration, unnicked single-chain BoNT E was injected (arrow) into a presynaptic neuron (to a calculated intracellular concentration of ~50nM). No inhibition of ACh release was observed even after 18h.
D. Unnicked BoNT E (25nM final, hatched area) was bath-applied to a presynaptic neuron preinjected with HC_A (arrow, intracellular concentration ~10 nM); the resultant decrease in response amplitude shows that unnicked BoNT E can enter the neuron and together with HC_A block ACh release.
E. Preinjection of H_2L (arrow, intracellular concentration ~100nM) into a presynaptic cell followed by bath application of unnicked BoNT E (25nM final, hatched area) gave no decrease in the release. Adapted from Poulain <u>et</u> <u>al.,</u> (1990); (J. Biol.Chem. in press).

Fig. 7. <u>Effect</u> <u>of</u> <u>BoNT</u> <u>chains</u> <u>and</u> <u>H_2L</u> <u>fragment,</u> <u>alone</u> <u>or</u> <u>in</u> <u>combination,</u> <u>on</u> <u>transmitter</u> <u>release</u> <u>in</u> Aplysia <u>neurons</u>
    ACh release was evoked in the buccal ganglion by a presynaptic action potential and the post-synaptic response that ensued recorded as a percentage of the control against time.
A. Intracellular injection (arrow) of H_2L into the presynaptic

cell (calculated intracellular concentration >10nM) did not change evoked ACh release. Subsequent addition to the bath (hatched area) of the HC_A (40nM), which enters the cell, inhibited transmission. Inserts represent recordings of action potential and post-synaptic responses (upper of each insert) 1h 40 min after injection of H_2L and 2h after bath addition of the HC_A (right hand side); note that in the latter the post-synaptic response is decreased whilst the presynaptic action potential remains unchanged. Calibration: vertical, 475 nS (response) and 50 mV (action potential); horizontal, 100 ms.
B. The LC from H_2L (at a calculated intracellular concentration of ~20nM) was injected (arrow) into the presynaptic neuron without modification of ACh release. A decrease in release was observed only upon addition to the bath (hatched area) of the HC_A (40 nM).
A. and B. from Poulain et al. (1989).
C. HC_A was injected (arrow) into the presynaptic cell, followed by bath application (hatched area) of LC_A; no change ensued. The schematic diagram shows the arrangement of the cells and sites of toxin administration.

Fig. 8. LC requires HC for its uptake in Aplysia neurons
        In all experiments, ACh release was evoked in the buccal ganglion of Aplysia by a presynaptic action potential and amplitude of the responses was recorded in the post-synaptic voltage-clamped neuron. These were expressed as a percentage of the control values and plotted as a function of time.
A. After control measurements were made, LC of type B was bath-applied (upper hatched area) at 40nM final concentration without effect on the evoked release. Subsequent addition (after 1h) of HC (40nM final concentration, lower hatched area) to the extracellular medium induced a decrease in ACh release; removal of the toxin chains by washing did not result in a recovery.
B. After stabilisation of the control recordings, HC of BoNT B (40nM final concentration, first hatched area) was bath applied for 1h and no effect on release ensued. After the HC was removed by extensive washing (75 min), the addition of LC of type B (40nM final concentration, second hatched area) to the bath resulted in a blockade of release.
C. Successive application of LC type A (10nM final concentration, first hatched area) and of HC type B (10nM final concentration, second hatched area) into the extracellular medium induced an irreversible decrease in ACh release. This demonstrates that HC_B can internalize LC_A and shows the heterologous chains are active together.
A.-C. are from Maisey et al. (1988).
D. Inclusion of a high concentration of LC_A in the bath followed by washing and addition of HC_A proved ineffective; presumably LC, unlike HC (cf B) is unable to bind to the neuronal membrane, and is, thus, removed by washing.

Fig. 9. Action of BoNT A and its chains on neuromuscular transmission
A. HC and LC exhibit low potency in blocking nerve-evoked muscle twitch tension. The individual chains of BoNT A were dialyzed into physiological medium and bath-applied to mouse phrenic nerve hemidiaphragms which were superfused at 23°C and stimulated supramaximally. Nerve-evoked muscle-twitch tension, expressed as a percentage of the initial value, was measured against time. HC (●) or LC (O) applied separately at 30nM failed to produce any change. In experiments involving both HC and LC (▲), super-

fusion was stopped, 30nM HC was bath-applied at time zero
followed after 10 min by the addition of 30nM LC; after a further
10 min superfusion was restarted. For comparison, the time
course of paralysis produced by 0.1nM ($\square$) BoNT A is also shown.
The results presented were obtained using two different prepara-
tions of the chains. Data points shown are the average of
duplicates; error bars indicate the range.
B. Inability of HC of BoNT A to prevent the blockade by intact
toxin of neuromuscular transmission. A relatively large excess
of HC was incubated with mouse phrenic nerve hemi-diaphragms,
under conditions designed to minimise its internalization,
followed by simultaneous exposure to BoNT A. After removal of
unbound toxin samples by washing, nerve-evoked muscle-twitch
tension, expressed as a percentage of the initial value, was
measured against time. For the pre-equilibration, the
temperature of the medium was reduced to 4°C and the tissue
incubated with a physiological solution differing from normal
Ringer in its concentration of Ca^{2+} (0.5mM) and Mg^{2+} (5mM).
Following further washing with the latter, HC (30nM) was applied;
after 30 min at 4°C BoNT A was added at a final concentration of
0.3nM. After further incubation at 4°C for either 15 or 30 min,
the tissues were washed repeatedly with the same buffer followed
by extensive washing with normal Ringer solution. The
temperature was then raised to 24°C, stimulation was initiated
and paralysis times determined. Incubation conditions used were:
0.3nM BoNT A for 15 min at 4°C following a 30-min pre-incubation
with 30nM HC ($\blacksquare$) or buffer alone ($\square$); 0.3nM BoNT A for 30 min
at 4°C after pre-equilibration for 30 min with 30nM HC ($\bullet$) or
buffer only ($\bigcirc$). The representative curves shown, obtained from
experiments run in parallel, were reproduced with three different
preparations of HC. From Maisey et al. (1988).

Fig. 10. Restoration of BoNT activity with a mixture of H_2L and HC

In both experiments, ACh release was evoked by presynaptic
action potentials and the subsequent responses (% control)
plotted against time.
A. Control responses evoked by the stimulation of two equivalent
presynaptic neurons afferent to the same postsynaptic neuron were
first recorded (O, $\bullet$). Addition of 40nM H_2L to the bath (hatched
area) which, alone, did not affect ACh release (see neuron O)
induced a decrease in release when the presynaptic neuron ($\bullet$) had
been pre-injected with HC_A (arrow, calculated intracellular
concentration > 10nM). This shows that H_2L is not sufficient to
inhibit release but is internalized and can act in conjunction
with HC_A to reduce ACh release.
B. After stabilization of control recordings, bath application
(first hatched area) of H_2L (100nM) did not modify the evoked
release. After the removal of H_2L by extensive washing of the
preparation, addition of HC_A (40nM, second hatched area) induced
the blockade of synaptic transmission. From Poulain et al.
(1989).

Fig. 11. Effects on neurotransmission in Aplysia neurons of
sequential application of HC, LC and H_2.
In both experiments, the amplitude (nS) of evoked postsynap-
tic responses was plotted against time.

A. Changes in the postsynaptic response resulting from the
presynaptic stimulation of a neuron ($\bullet$) injected with HC (arrow,
intracellular concentration ~10nM) were compared with those of a

second, non-injected (O) presynaptic cell, afferent to the same postsynaptic neuron. After bath application of LC_A (40nM, hatched area), addition to the bath (dashed area) of H_2 (40nM) induced a depression of neurotransmitter release only in the cell injected with HC.
B. A presynaptic neuron was injected with HC_A (arrow, calculated intracellular concentration ~10nM), then H_2 was bath-applied (25nM) without any change in transmission, showing that this fragment was not contaminated with H_2L. Subsequent bath application of the LC (25nM) led to expression of toxicity.
Typical recordings of action potentials (lower) and responses (upper) are shown just after injection of HC and, in presence of H_2, 80 min after bath application of the LC; in the latter, only the post-synaptic response is altered as expected. Calibration: vertical, 600 nS (postsynaptic response) and 50 mV (action potential); horizontal, 75 ms. From Poulain et al. (1989).

Fig. 12. Autoradiograms of SDS electrophoresis gels showing ADP-ribosylation of brain synaptosomal proteins with BoNT type D preparation, pertussis and cholera toxins
       After gradient polyacrylamide (5-20% w/v) electrophoresis of synaptosomal samples, performed under reducing conditions, the gels were dried and autoradiograms prepared.
A. Lysate of rat cerebrocortical synaptosomes were labelled for 2h at 30°C using 15uM ^{32}P-NAD and 10mM dithiothreitol in buffer containing 60 ug/ml type D toxin (track 1), 200nM BoNT A (2), or B (3), 25 ug/ml activated pertussis toxin (4) or 62.5 ug/ml cholera A1 subunit (5).
B. ADP-ribosylation was carried out with 60 ug/ml type D toxin (tracks 1-4) and 25 ug/ml activated pertussis toxin (tracks 5 & 6) using lysates from synaptosomes that had been pre-incubated for 90 min at 37°C in the absence of any toxin (tracks 1 & 5), and with the inclusion of 200nM BoNT A (2), B (3) or 60 ug/ml D (4) or 2 ug/ml pertussis toxin (6).
C. Synaptic vesicles (40 ug/sample), purified from cerebrocortical synaptosomes, were pre-incubated for 15 min at 30°C with type D (track 1), BoNT A (2) or B (3) or activated pertussis as in (A).
After addition of ^{32}P-NAD (15 uM), the samples were incubated for 2h before electrophoresis/autoradiography as above. Note that gels in A, B and C are from separate experiments. Adapted from Ashton et al. (1988).

Fig. 13. Effect of botulinum toxins on synaptosomal uptake and Ca^{2+}-dependent K^+-stimulated release of [^{3}H]-noradrenaline
       Synaptosomes were incubated for 90 min at 37°C with 0.1 uM [^{3}H]noradrenaline, in the absence or presence of toxin; after washing, transmitter release was measured over a 5 min period.
Values of uptake represent the sum of the radioactivity released into the supernatant together with that remaining inside the synaptosomes. Uptake (open bars) and release (hatched bars) are expressed relative to the value for the non-toxin control; release represented 4.0±0.2% of the total transmitter present which was 260,000±19,000 dpm/mg of synaptosomal protein. Columns: 1, non-toxin treated control; 2, 1 ug/ml type D toxin; 3, 60 ug/ml type D toxin; 4, 200nM BoNT A and 5, 200nM BoNT B. Values shown are the mean±the range of 2 independent experiments done in duplicate

Fig. 14. **Relative distribution of synaptophysin, low M_r G-proteins and ras p21 in purified synaptic vesicles from bovine brain**
A partially purified sample of vesicles (p3 fraction; 93mg protein) was subjected to gel permeation on a Sephacryl S-1000 column (2.8 x 90cm) and 8ml fractions collected. After SDS polyacrylamide gel electrophoresis in the presence of ß-mercaptoethanol, the fractions were analyzed by immunoblotting using antibodies to p38 (A), a-^{32}P-GTP binding monitored by autoradiography (B) or immunoblotting with anti-ras p21 anti-bodies (C). Arrowheads show the mobilities of 3 GTP-binding bands whilst arrows on left side indicate positions of standard protein markers. From Matsuoka and Dolly (1990; submitted).

Fig. 15. **Autoradiograms of SDS gels showing the inability of BoNT to affect phosphorylation of synaptic vesicle proteins**
Electrophoresis/autoradiography was carried out as in Fig. 12.
A. Synaptosomes purified from rat cerebral cortex were incubated for 90 min at 37°C in the absence (tracks 1 & 3) or presence of 200nM BoNT A (tracks 2,4). After toxin removal by washing, the nerve terminals were depolarized with 25mM KCl for 20 sec prior to lysis and preparation of synaptic vesicles by density gradient centrifugation. The resultant samples were incubated with 10uM ^{32}P-ATP (5 uCi) for 1 min prior to exposure to SDS electro-phoresis buffer. Tracks 1 & 3 show phosphorylation in basal buffer whilst 2 & 4 contained 0.3mM Ca^{2+} plus 4uM calmodulin.
B. A synaptosomal suspension was labelled for 90 min with 2.5uM ^{32}Pi (0.3 mCi/ml) in the absence (track 1) or presence (2) of 200nM BoNT A. After washing, the synaptosomes were depolarised for 20 sec in 25mM KCl in Ca^{2+}-containing buffer. The samples were then lysed in medium containing a 'cocktail' of kinase and phosphatase inhibitors, vesicles isolated by differential centrifugation and analysed electrophoretically. The numbers shown in A and B give the M_r (kDa) of phosphorylated proteins described in the literature. Adapted from Ashton et al., (1988a).
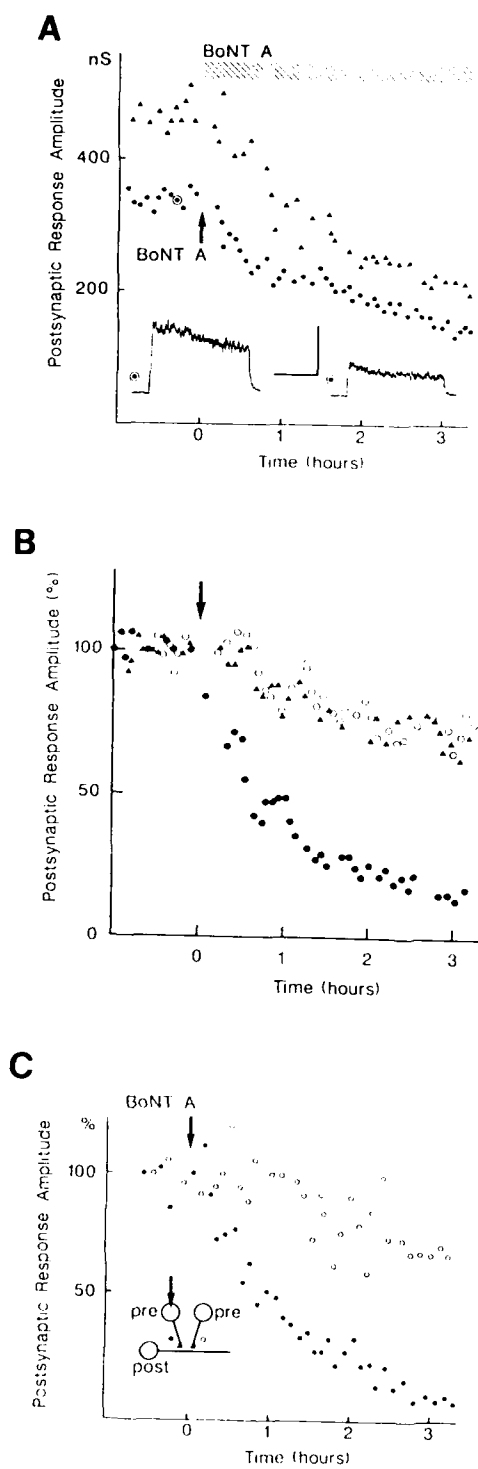
FIG. 1

FIG. 2

A

nS

BoNT A

BoNT A

400

200

Postsynaptic Response Amplitude

0   1   2   3

Time (hours)

B

Postsynaptic Response Amplitude (%)

100

50

0

0   1   2   3

Time (hours)

C

BoNT A

%

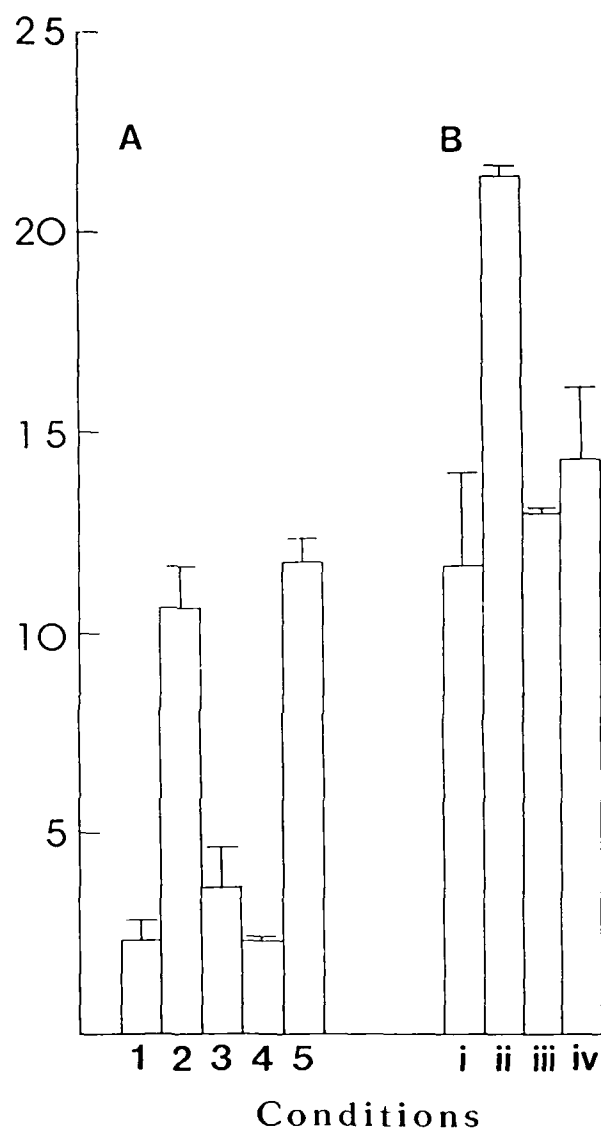Postsynaptic Response Amplitude

100

50

pre   pre

post

0   1   2   3

Time (hours)

FIG. 3

FIG. 4

FIG. 5.

FIG. 6 -

FIG. 7

FIG. 8

FIG.9

FIG. 10

FIG.11

FIG. 12

FIG. 13

FIG. 14

FIG. 15